# EVIDENCE FOR THE PROTEIN NATURE OF THE SPERM AGGLUTININS OF THE KEYHOLE LIMPET AND THE SEA-URCHIN

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Chemical investigations on agglutinins (both naturally occurring and immune) for blood cells and bacteria have furnished strong evidence for the view that they are always of protein nature (see Marrack, 1938). It might be expected, then, that the sperm agglutinins present in the egg water of certain species of marine animals (see Lillie, 1919; Lillie and Just, 1924; Just, 1930; Tyler, 1940) would also show protein properties. This was not found to be the case by the earlier investigators. Glaser (1914) and Woodward (1918) applied a number of common protein tests to sea-urchin (*Arbacia*) egg water and obtained no reaction with the exception of a weak, partial xanthoproteic test. This does not, however, eliminate the possibility that the agglutinin is of protein nature, since it has often been shown that physiological responses can be evoked by solutions of proteins too dilute to give the ordinary tests. In fact, proteins can be detected in serological reactions at dilutions at which the color tests fail. For example, Uhlenhuth (1909) showed that a dilution of a protein as high as 1 to 100,000 will give a detectable reaction with anti-serum whereas neither the Biuret nor the Millon's reaction will exceed 1 to 10,000. Agglutination reactions are many times more sensitive than precipitin reactions (Zinsser, 1939; p. 246) and anaphylactic reactions still more sensitive.

In a preliminary note (Tyler and Fox, 1939) evidence that the sperm agglutinins of the keyhole limpet and of the sea-urchin are proteins has been reported. The present article presents the details of this evidence and further information on the properties and possible methods of purification of the agglutinins.

## Bioassay

The agglutination reaction in the keyhole limpet, *Megathura crenulata*, has recently been described (Tyler, 1940*a*) in some detail. It was shown that the time at which agglutination of the sperm first becomes macroscopically visible increases with increasing dilution of the egg water (agglutinin), within certain limits and for a given sperm

suspension. This relation between concentration and agglutination time can be employed in a bioassay. For this purpose an arbitrary agglutination time is taken as a standard and the dilution of the test solution that gives the standard agglutination time is determined. The reciprocal of the dilution factor gives the agglutinin titer in arbitrary units. Since with concentrated solutions the agglutination time changes very much less than with dilute solutions, it is advisable to take as a standard a reaction obtained in a relatively dilute solution. We have usually taken as a standard a reaction that is first visible macroscopically in 30 seconds when equal volumes of the agglutinin solution and a 1 per cent sperm suspension are mixed. Solutions giving the standard reaction time are designated as having one unit concentration of agglutinin. The unit concentration selected is well above the weakest solution that gives a perceptible reaction. The latter end point, however, is not as easily determined. Sperm suspensions from different animals as well as suspensions of different ages and history from the same animal will, of course, show considerable variation in their reaction time even when employed in closely similar concentrations. The units have only approximate significance when comparisons of different series of tests are made. In most instances, however, the comparisons are made between control and test solutions simultaneously on samples of the same sperm suspensions. The concentration of agglutinin in the test solution is then given as a fraction or percentage of that in the control.

Other methods of bioassay that were tried with the keyhole limpet included determining the number and size of the clumps of agglutinated sperm and centrifuging in a hematocrit tube. These two methods did not prove as convenient nor as reproducible as the method of determining the time for agglutination to become macroscopically visible.

The agglutination reaction in the sea-urchin, *Strongylocentrotus purpuratus* (Loeb, 1914; Lillie, 1921), is quite similar to that in *Arbacia* (Lillie, 1913). The reaction occurs so rapidly even with dilute agglutinin solutions that it is not feasible to use the time at which it first becomes visible for bioassay. The method employed by Lillie (1914) is preferable. He showed that in the sea-urchin the agglutination reverses (i.e. the sperm disperse) within a short period of time ranging from a few seconds to a few minutes. The time at which reversal occurs decreases with decreasing concentration of the egg water. Lillie defined as a unit a solution of such concentration that the reaction reverses within three to five seconds, the observations being made under the microscope. For greater convenience we employed, as a standard, a reaction that reverses in one minute, the observations

being made macroscopically. A solution which, when mixed with an equal volume of a 1 per cent sperm suspension, gives a reaction that lasts one minute, is then designated as having one unit concentration of agglutinin.

## Extraction

As Lillie first showed with *Arbacia* and *Nereis*, it is unnecessary to treat the eggs in any special manner in order to obtain agglutinin solutions. The sea water above the eggs becomes charged with the substance, the concentration increasing continuously with time. Allowing eggs to age in sea water is, however, not a very satisfactory method for obtaining solutions of high agglutinin titer. The eggs slowly disintegrate as they remain in sea water. This not only encourages bacterial growth and adds to the impurities but also liberates an anti-agglutinin (Lillie, 1914; Tyler, 1940*b*).

The method we have employed consists simply in dissolving the jelly layer surrounding the egg by means of acidified sea water. As was shown in the previous article, the agglutinin is either the jelly substance itself, or a component of it. The jelly slowly dissolves as the eggs age in ordinary sea water. In the sea-urchin, sea water acidified to pH 3.5 almost immediately dissolves the jelly layer without injuring the rest of the egg. A single extraction gives a solution containing practically all of the agglutinin that can be obtained from the eggs. A suspension of about $10^8$ eggs in 100 cc. of acid sea water gives a solution of 32 units. Different preparations, however, vary widely in the yield.

In the keyhole limpet, the jelly layer does not dissolve as rapidly in acidified sea water as in the sea-urchin. There is, however, a rapid swelling and softening of the jelly and the eggs can be readily centrifuged out of their jelly hulls in the acid sea water. By this means agglutinin solutions of very high titer are rapidly obtained in the keyhole limpet too.

The agglutinins can also be rapidly extracted with isotonic NaCl acidified to pH 3.5. This was employed in some experiments in which it was desirable to eliminate certain of the sea water salts.

Rather weak or inactive preparations are obtained by such methods as freezing and thawing or extraction with distilled water which entail cytolysis of the eggs. This is due to the inactivation of the agglutinin by an anti-agglutinin (recently isolated by Tyler, 1940*b*) present within the eggs.

Extraction of the eggs by alcohol, acetone or ether failed to give active preparations. Also the agglutinin was not extractable from active precipitates (see below) or solutions by means of these solvents.

## Dialysis

Lillie (1914) showed that the sperm agglutinin of *Arbacia* is non-dialyzable. We have found the same to be true for the sperm agglutinins of *Strongylocentrotus* and of *Megathura*. Concentrated solutions (approximately 20 to 30 units) of the agglutinins were placed in cellophane tubes or in collodion bags and dialyzed against approximately two to three times the volume of ordinary sea water with constant stirring. The dialysis was carried out in a cold room at 1° C. Samples were tested at regular intervals up to two weeks. The dialysate in all cases was completely inactive while the solutions within the tubes or bags retained practically their original activity during this period. Other samples that were dialyzed against running sea water likewise showed no loss in activity. It is evident, then, that the sperm agglutinins of the sea-urchin and of the keyhole limpet are substances of large molecular size.

## Precipitation

It has been reported by Woodward (1918) that the sperm agglutinin of *Arbacia* partially precipitates when the egg water is saturated with $(NH_4)_2SO_4$. In *Strongylocentrotus* and *Megathura* we find that the agglutinins can be practically completely precipitated by the addition of $(NH_4)_2SO_4$.

When concentrated egg water of *Strongylocentrotus* or of *Megathura* is saturated with $(NH_4)_2SO_4$ a white flocculent precipitate slowly appears. The precipitation is usually complete within about 24 hours. The initial egg waters are usually opalescent in appearance. After removal of the precipitates the supernatants appear quite clear. Precipitates obtained from *Strongylocentrotus* and from *Megathura* egg waters were dissolved in sea water, dialyzed against running sea water to remove the $(NH_4)_2SO_4$, and adjusted to the original volumes. Agglutination tests with these solutions showed in all cases no significant differences from the original solutions. The supernatant solutions were also dialyzed and tested. With none of the latter was any sign of agglutination obtained. The following table gives the results of three such experiments with the keyhole limpet and two with the sea-urchin. The figures represent units concentration of agglutinin as defined above (p. 154).

| | *Megathura* | | | *Strongylocentrotus* | |
|---|---|---|---|---|---|
| | *A* | *B* | *C* | *A* | *B* |
| Original egg water | 16 | 8 | 6 | 32 | 24 |
| Precipitate | 12 | 10 | 8 | 32 | 20 |
| Supernatant | 0 | 0 | 0 | 0 | 0 |

It is evident that, in both *Megathura* and *Strongylocentrotus*, the agglutinin is completely salted out by saturation with ammonium sulfate.

An approximate determination was made, with *Megathura* egg water, of the lowest concentration of ammonium sulfate required for complete salting out of the agglutinin. To four 100-ml. portions of an approximately 10 unit agglutinin solution, made up in isotonic (0.55M) NaCl at pH 3, were added 30, 40, 50 and 55 grams of ammonium sulfate respectively. The salt dissolved completely in the first three and a small amount remained undissolved in the fourth. After standing 24 hours at room temperature the second, third and fourth flasks showed flocculent precipitates and clear supernatant solutions. The first flask showed no precipitate and the solution remained opalescent. The agglutinin titers of the precipitate and the supernatant in the second flask (40 grams) were determined after dialysis and adjustment to original volume. The precipitate gave practically the same agglutinin titer as the original solution; the supernatant showed no activity. Precipitation of the agglutinin is, then, complete in approximately three-fourths saturated ammonium sulfate.

A preliminary attempt at fractionation was made by slowly increasing the concentration of ammonium sulfate and removing the precipitate that first appears (at about 2/3 saturation) separately from that which comes out at higher saturation. Both fractions showed activity and, based on the relative centrifuge volumes of the precipitates, no marked difference in agglutinin titer was manifest.

Preliminary attempts at crystallization were also made with material prepared by dialysis, adsorption (see below), and repeated precipitation with 2/3 to 3/4 saturated ammonium sulfate by allowing the precipitation to take place in a dialyzing bag immersed in a solution of ammonium sulfate, the concentration of which was very slowly increased; also by slow evaporation of ammonium sulfate solutions of the agglutinin. In none of these were recognizable crystals obtained.

## Adsorption

The agglutinins of both *Megathura* and *Strongylocentrotus* can be completely removed from solution by solid $CaCO_3$. For elution the $CaCO_3$ is dissolved by the addition of acid. This involves the disadvantage that some or all of the agglutinin may be inactivated by the acid (see below). To avoid this, it is necessary to employ a concentration of acid just sufficient to dissolve the $CaCO_3$ at a reasonable rate but not strong enough to inactivate the agglutinin. By use of sea water acidified to about pH 2.5 to 2.8 we have recovered approximately 25 to 50 per cent of the original agglutinin. Other methods of elution have not as yet been investigated.

The agglutinins are also adsorbed by $Al_2O_3$, charcoal and kaolin.

## Color Tests

Several of the common color tests for proteins were applied to concentrates of sea-urchin and keyhole limpet agglutinins. The material was prepared by dialysis and ammonium sulfate precipitation of egg water obtained with the utmost care to avoid injury to the eggs themselves. In the case of the sea-urchin, it is quite feasible to prepare a concentrated egg water without any injury to the eggs and also to avoid or get rid of body fluid contamination. In the keyhole limpet this is more difficult unless one employs naturally shed eggs (see previous article). Definitely positive xanthoproteic, biuret and Millon's reactions were obtained with the agglutinin preparations from both sea-urchin and keyhole limpet. For the Millon's test it is necessary to dialyze against distilled water (washing the $(NH_4)_2SO_4$ precipitate on a membrane filter suffices) in order to remove salts, particularly chlorides, that interfere with the reaction. While the distilled water generally inactivates the agglutinin, this does not by any means invalidate the test as an indicator of the presence of protein. Attempts were also made to obtain sugar tests on portions of the material heated in concentrated HCl. These were all negative.

The failure of the earlier workers (Glaser, 1914; Woodward, 1918) to obtain positive protein tests with *Arbacia* egg water may very likely be due to their having worked with much weaker preparations. Positive reactions to these color tests do not, of course, prove that the agglutinin is of protein nature. They do, however, show the presence of proteins in the preparations. Since the method of obtaining the egg waters involves practically no destruction of the eggs themselves but simply dissolves the jelly layer, it is unlikely that there are very many different substances of high molecular weight present in the preparations. Previous evidence (Tyler, 1940*a*) had shown that the agglutinin is either the jelly substance itself or if the jelly is composed of more than one substance, that it is a component of it. If it were known that only a single high molecular weight substance were present in the preparations, then the color tests would be convincing evidence of the protein nature of the agglutinin. In place of such information other kinds of evidence have been obtained.

## Action of Enzymes

Evidence for the protein or polypeptide nature of a substance may be obtained by digestion with proteolytic enzymes. The evidence is not very satisfactory when crude preparations of the proteinases are

employed since in such preparations there are generally other enzymes present. In recent years, however, a number of proteolytic enzymes have been crystallized (see Northrop, 1939), and these are, of course, eminently suitable for the tests. We have obtained, through the courtesy of Dr. J. H. Northrop, samples of crystalline trypsin and chymotrypsin and have examined their action on the sperm agglutinins of the keyhole limpet and the sea-urchin.

The *Strongylocentrotus* agglutinin is inactivated fairly rapidly by both trypsin and chymotrypsin. In four experiments that were run, inactivation was practically complete in less than 3 hours. The following figures give the agglutinin titers in one of the experiments in which solutions of trypsin, chymotrypsin and also steapsin (commercial) all adjusted to pH 8.0 were added to equal volumes of egg water, allowed to act at 20° C. and samples tested at the times indicated.

| | 2 min. | 1 hour | 2 hours | 3 hours |
|---|---|---|---|---|
| Control | 8 | 8 | 8 | 8 |
| Saturated trypsin | 8 | 6 | 3 | 0 |
| 1 per cent chymotrypsin | 8 | 1/2 | 0 | 0 |
| Saturated steapsin | 8 | 8 | 6 | 6 |

The slight inactivation obtained with the commercial steapsin is perhaps to be attributed to small amounts of other enzymes present as impurities. The inactivation of the agglutinin by trypsin and by chymotrypsin occurs more rapidly than might be expected on the basis of the rate at which proteins are in general hydrolyzed by these enzymes. However, it is not necessary to assume that the substance must be split by the enzymes in order for inactivation to occur. In enzyme reactions there is generally considered to be an intermediate addition compound first formed. If, in the case of the agglutinin, the initial combination involved those groups that are concerned with its reaction with the sperm, there would be, in the presence of sufficient enzyme, a very rapid inactivation.

The *Megathura* agglutinin is much more slowly inactivated by solutions of the crystalline proteinases. In three experiments that were run, about seven days were required to reduce the titer to a point where no definite agglutination reaction is obtained. It is important, therefore, to have reasonably sterile conditions. This is relatively easy to do in the case of the keyhole limpet agglutinin since the solutions can withstand boiling for a considerable time (see below). The following figures (agglutinin concentration units—see bioassay section) give the course of the inactivation in one experiment run at pH 8 and 20° C.

| | 5 min. | 1 day | 2 days | 3 days | 5 days | 7 days | 8 days |
|---|---|---|---|---|---|---|---|
| Control | 16 | 14 | 16 | 16 | 14 | 12 | 12 |
| Saturated trypsin | 16 | 14 | 12 | 12 | 10 | 1/2 | 0 |
| 1 per cent chymotrypsin | 16 | 16 | 10 | 6 | 1/2 | 0 | 0 |

In this, as well as the other two experiments, the inactivation sets in slowly during the first few days and then proceeds more rapidly thereafter. Assuming that here, too, the initial combination of enzyme with substrate is quite rapid, it appears that this does not involve those groups on the agglutinin molecule which enable it to react with the sperm. The inactivation must then occur during the actual digestion of the agglutinin.

It would be desirable to determine whether or not there is a difference between the sea-urchin and the keyhole limpet agglutinins in regard to their manner of inactivation by these proteolytic enzymes. This could be tested by examining the digests for products of hydrolysis during the course of the inactivation. Due to limitations of material and to the desirability of using preparations that are of known purity for such purposes, the determinations have not as yet been made. One set of formol titrations has, however, been run on the digests (and controls) of the keyhole limpet agglutinin in the experiment listed above. The results showed that appreciable hydrolysis had occurred in the enzyme solutions. The determinations were made on aliquot samples removed on the tenth day. Solutions of trypsin and of chymotrypsin that had been kept under the same conditions as the digestion mixtures were added to samples of the control agglutinin solution at that time and the digestion mixtures were diluted correspondingly with sea water. Allowing for the controls the formol titrations gave $8 \times 10^{-6}$ equivalents of $-COOH$ per ml. for the trypsin digest and $13 \times 10^{-6}$ equivalents for the chymotrypsin digest.

## Heat and pH Inactivation

The stability of solutions of both the keyhole limpet and the sea-urchin agglutinins varies with the temperature and the pH at which they are kept. Since the time for inactivation at a given temperature is a function of the pH of the solution, both of these variables may be considered together.

The sea-urchin agglutinin is fairly rapidly inactivated by heating the solutions. The following figures give the time required for half-inactivation and for practically complete inactivation of solutions of various pH at 100° C.

| pH | 2.50 | 3.1 | 4.2 | 6.3 | 7.3 | 8.2 |
|---|---|---|---|---|---|---|
| Minutes for 50 per cent inactivation | ..... | 2–3 | 12–15 | 15–20 | 6–8 | 4–5 |
| Minutes for 95–100 per cent inactivation | 3 | 5–7 | 20–25 | 70–85 | 20–30 | 15–20 |

As the figures show, the agglutinin is most stable in the range of pH 4 to 7. At lower temperatures the time for inactivation increases, but the effect of pH remains substantially the same. Thus at 20° C. solutions at pH 4 retain their full activity for more than three days while pH 2 and pH 8 solutions are completely inactivated. At 0° C. solutions at pH 4 have been kept for over 6 months with no appreciable loss in activity.

When sea water solutions of the agglutinin are made alkaline (pH 9 and above), a precipitate of calcium and magnesium carbonates and hydroxides forms and this adsorbs the agglutinin. The agglutinin can be completely recovered by dissolving the precipitate in acid sea water. Due to the formation of the precipitate and adsorption of the agglutinin, sea water solutions could not be used for determining the rate of inactivation in the more alkaline range. Solutions in isotonic NaCl were employed for this purpose and these showed a continuous decrease in stability as the pH was raised.

The keyhole limpet agglutinin is considerably more stable in solution than is that of the sea-urchin. The following figures give the time required for half-inactivation and for nearly complete inactivation of solutions of various pH at 100° C.

| pH | 1.2 | 2.0 | 3.2 | 5.0 | 8.2 | 9.2 | 11.0 |
|---|---|---|---|---|---|---|---|
| Hours for 50 per cent inactivation | 1/6 | 1–1½ | 24–36 | 21–30 | 5–9 | 4–6 | 1/30 |
| Hours for 95–100 per cent inactivation | 1/2 | 3–3½ | 60–90 | 44–90 | 14–18 | 14–18 | 1/6 |

The determinations at pH 9.2 and 11.0 were made on solutions of the agglutinin in isotonic NaCl, since, as in the case of the sea-urchin, the precipitate that forms upon the addition of alkali to sea water adsorbs the agglutinin. Here again the agglutinin can be completely recovered by dissolving the precipitate. The pH range of maximum stability of the keyhole limpet agglutinin is roughly the same as that of the sea-urchin. It is, however, considerably more stable, being able to withstand boiling for more than 24 hours with only a 50 per cent loss in activity. At lower temperatures the stability varies in the

same manner with pH. Solutions of pH 2.5 to 5.5 kept at room temperature remain fully active for more than a week while solutions at pH 1 and pH 11 are completely inactivated in less than a day. At 0° C. the solutions of pH 2.5 to 5.5 have been kept more than 6 months with no loss of activity.

The inactivation of agglutinin solutions by heat may be considered to be due to a denaturation of the active substance. With concentrated solutions of both the keyhole limpet and the sea-urchin agglutinins a precipitate forms upon inactivation by heating. It is possible, however, to inactivate completely the agglutinins without the appearance of a precipitate or coagulum. This occurs generally with dilute solutions. The failure of a coagulum to appear upon inactivation of dilute solutions does not, however, exclude the possibility that the effect is due to denaturation of the substance. Denaturation is generally assumed to involve more than one step (see Mirsky, 1938), the final one being coagulation.

## Dry Weights and Nitrogen Content

The concentration of organic matter was determined in agglutinin solutions of keyhole limpet and of sea-urchin prepared with special care to avoid injury to the eggs and contamination with body fluids, etc. Samples of the solutions were dialyzed against distilled water of known solid content, evaporated to small volume by boiling and dried at 80° C. to constant weight. Micro-Kjeldahl nitrogen determinations were then made on the dried material. The results were as follows:

| | | *Megathura* | *Strongylocentrotus* |
|---|---|---|---|
| Titer of Solutions | | 32 units | 16 units |
| Organic solid content | range | 0.10–0.14% | 0.028–0.031% |
| | average | 0.11% | 0.03% |
| Nitrogen content | range | 2.8–5.2% | 3.8–5.9% |
| | average | 4.6% | 5.2% |

The low content of organic solid matter may perhaps account for the failure of the earlier workers to obtain positive protein tests on sea-urchin agglutinin. The nitrogen content (about 5 per cent) is evidently too low for a pure protein. However, we do not as yet know how much inactive material may be present. The present values may be taken then merely as showing the presence of a definitely detectable quantity of nitrogen which is consistent with the other evidence that the agglutinin is of protein nature.

The amount of solid matter contributed per egg may be estimated roughly for the sea-urchin from the fact that a 16 unit solution is ob-

tained by extracting $5 \times 10^7$ eggs in 100 cc. of acid sea water. Since 100 cc. of the solution yields 30 mg. of organic solid matter, a single egg contributed $6 \times 10^{-7}$ mg. In the absence of information as to the amount of inactive material present, this value may be taken as representing a maximum for the quantity of agglutinin obtainable per egg. The volume of an egg of *S. purpuratus* is $2.6 \times 10^{-7}$ cc. and, assuming a density of 1.04, the wet weight would be $2.7 \times 10^{-4}$ mg. From the figures of Leitch (1934), the dry weight would be about one-fifth of the wet weight or $5 \times 10^{-5}$ mg., and roughly three-fourths of this is protein. The quantity of agglutinin obtained from a single egg would then correspond at most to 1 per cent of the dry weight of the egg.

## Discussion

From the method of extraction, the non-dialyzability, precipitation with $(NH_4)_2SO_4$, the color tests, the heat- and pH-lability, the insolubility in alcohol and ether, the presence of nitrogen and particularly the inactivation by means of purified proteinases, one may conclude that the sperm-agglutinin both of the sea-urchin and of the keyhole limpet is either protein or closely associated therewith. This is consistent with the results on other naturally occurring agglutinins as well as immune agglutinins (and antibodies in general) that have been investigated (see Marrack, 1938; Heidelberger, 1938; Landsteiner, 1936; Zinsser, 1939). It is also in line with the specificity of the reaction.

The other active substances that have been isolated from sperm and eggs: namely, the anti-agglutinins, egg agglutinins and egg membrane lysin (Tyler, 1939, 1940*b*), also appear to be of protein nature. It would seem, then, that there is some justification for the analogies which Lillie drew between the fertilization reaction and immunological reactions. However, considerably more work will be necessary in both fields before we can determine to what extent immunological principles may be used to interpret the fertilization reactions.

The sperm agglutinins of the two animals investigated differ markedly, as the results show, in certain properties. That of the keyhole limpet is considerably more resistant to inactivation by heat (and pH change) and by proteolytic enzymes than is that of the sea-urchin. The situation is, however, not unusual. Marrack (1938, p. 50) reports that the heat stability of agglutinins (and antibodies in general) is very variable. Even in the same sera considerable differences in lability between different agglutinins have been found. Different antibodies also differ in regard to their resistance to the action of proteolytic enzymes (Marrack, p. 52). The destruction proceeds in

many cases quite slowly and in some instances use has been made of the relative resistance to digestion in attempts to purify antibodies (see Zinsser, p. 172).

The difference in stability between keyhole limpet and sea-urchin agglutinins correlates with differences in the nature of the reaction in these two forms. It has been previously shown (Tyler, 1940*a*) that the sperm agglutination reaction in the keyhole limpet persists considerably longer than does the reaction in the sea-urchin. Corresponding to the difference in duration of the reaction, a difference was found in the time at which precipitation occurs when the active principle (anti-agglutinin) from sperm is added to agglutinin solutions. In both species inactivation of the agglutinin occurs immediately after addition of the anti-agglutinin but while precipitation occurs within a few minutes in the sea-urchin it is many hours later in the keyhole limpet. These differences are not necessarily attributable entirely to the agglutinins, since the properties of the sperm and the sperm extracts cannot be assumed to be the same in the two species. Nevertheless, it is of interest to note that if greater stability is assumed to mean also slower reactivity on the part of the substance, then the differences in the properties of the keyhole limpet and sea-urchin agglutinins reported here afford a reasonable interpretation of the difference in the agglutination reaction in these two forms. In other words, the reactivity of the keyhole limpet substance may be considered to be lower than that of the sea-urchin.

## Summary

Some of the chemical and physical properties of the sperm agglutinin (fertilizin) of the keyhole limpet *Megathura crenulata* and of the sea-urchin *Strongylocentrotus purpuratus* were investigated. In both species the agglutinins were found to be non-dialyzable. They precipitate completely without loss of activity in nearly saturated ammonium sulfate. They are adsorbed by $CaCO_3$, $Al_2O_3$, charcoal and kaolin. The agglutinins are insoluble in alcohol and ether. Active concentrates give, contrary to the findings of earlier investigators, the common color tests for proteins and are found to contain nitrogen. Solutions of crystallized proteinases (trypsin and chymotrypsin) inactivate the agglutinins. They are also inactivated by heat as a function of pH. From the evidence it is concluded that in both species the agglutinating principle is either protein or very closely associated with protein.

The keyhole limpet agglutinin is much more resistant than is that of the sea-urchin to inactivation by heat and by proteolytic enzymes.

It is suggested that the difference between the two species in the duration of the agglutination reaction may be related to the difference in stability of the respective agglutinins.

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